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Interkingdom Communication: Study of Caenorhabditis elegans and Vibrio Cholerae Interactions

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INTERKINGDOM COMMUNICATION: STUDY OF *CAENORHABDITIS ELEGANS*
AND *VIBRIO CHOLERA*E INTERACTIONS

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Joseph T. Angeloni
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Accepted by:
Dr. Min Cao, Committee Chair
Dr. Yuqing Dong
Dr. Thomas A. Hughes

ABSTRACT

Vibrio cholerae is a Gram-negative, rod-shaped bacterium that is mainly found in water environments, and is the causative agent of the disease cholera in humans. It is critical for this bacterium to communicate via quorum sensing to persist and survive in the environment, as well as cause infection. Recently, it has been shown that eukaryotes are able to sense and respond to certain quorum sensing molecules, known as autoinducers, which are produced by different bacteria. *Caenorhabditis elegans* is a particularly useful model for studying this interaction. During preliminary experiments, it was noticed that *C. elegans* were strongly attracted to *V. cholerae* C6706 O1 El Tor, although this bacterium kills the nematodes at a high rate and significantly decreases lifespan. To further study this phenomenon, chemotaxis assays and lifespan assays were conducted using *C. elegans* N2 strain. Different *Vibrio* spp. were tested (*V. cholerae*, *V. harveyi*, and *V. fischeri*) to figure out what underlying molecules were driving chemoattraction behavior in *C. elegans*. It was found that *C. elegans* can sense various autoinducer molecules with preferences, but other signaling molecules also appear to be involved in chemotaxis behavior. Interestingly, the ability of *V. cholerae* to attract *C. elegans* N2 seems to be dependent on ToxT, which activates the transcription of virulence genes that are necessary for pathogenesis and leads to increased levels of cyclic GMP-AMP (c-GAMP), a recently identified new second messenger that has only been reported in *V. cholerae*. It is thus hypothesized that cyclic di-nucleotides, specifically c-GAMP and c-di-GMP, are playing an important role influencing host behavioral modifications. It has been shown that 1nM concentrations of both c-GAMP and c-di-

GMP are able to influence a positive chemotactic response in *C. elegans*. Through lifespan assays, killing of *C. elegans* by *V. cholerae* also appears to be dependent on ToxT, but not DncV, which is the cyclase responsible for the production of c-GAMP. This work will ultimately allow for a better understanding of the specific mechanisms involved in interkingdom communication, as well as shed light on how *V. cholerae* O1 El Tor is able to persist and cause disease within a host.

DEDICATION

I would like to dedicate this work to my friends and family who have helped me through my academic journey. My parents, Gary and Carol, who have always been there and supported me in whatever I planned to accomplish in life, have taught me valuable life lessons and continue to be the foundation to my success in the future. They will always be my source of inspiration. Also, I would like to thank my girlfriend Meghan Goodwin, her family, and my sister, Stephanie, who have been with me since I began my graduate work at Clemson University. Having a family so close, when it is difficult to travel a great distance to visit my own, has helped immensely and I value their guidance throughout my life.

ACKNOWLEDGEMENT

I would like to sincerely thank my advisor, Dr. Min Cao, for providing me with every chance to succeed while working in her lab and the opportunity to have an integral role in this study. This work would not have been possible if it wasn't for her exceptional guidance throughout and vast knowledge of the subject. I knew that I could always turn to her when I had a question, and she has continuously supported me since I joined her lab. I would like to thank my committee members, Dr. Yuqing Dong and Dr. Thomas Hughes, who have provided valuable input towards this project and have helped with numerous decisions during my time at Clemson.

I would also like to thank my lab mates for their insightful discussion, experimental advice and assistance in the lab: Miranda Klees, Daniel Pederson, Ojas Natarajan, Jessica Dinh, Ethan Wilson, XiaXiao Wang, and Hong Guo. Lastly I would like to thank Mikaela Conley, Phoebe Hourigan, and Megan Hunt for their initial trials on experiments and assistance provided in this work.

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LIST OF ABBREVIATIONS

| | |
|--------------------|--|
| AI | Autoinducer |
| AI-1 | Autoinducer-1 |
| AI-2 | Autoinducer-2 |
| C8-HSL | N-octanoyl-L-homoserine |
| CAI-1 | Cholera Autoinducer-1 |
| c-di-GMP | Cyclic Dimeric Guanosinemonophosphate |
| c-di-AMP | Cyclic Dimeric Adenosinemonophosphate |
| c-GAMP | Cyclic Guanosinemonophosphate – Adenosinemonophosphate |
| CT | Cholera Toxin |
| FUDR | Fluorodeoxyuridine |
| HAI-1 | <i>harveyi</i> autoinducer-1 |
| ILS | Insulin-Like Signaling |
| LB | Luria-Bertani Broth |
| NGM | Nematode Growth Medium |
| qRT-PCR | Quantitative Real-time Polymerase Chain Reaction |
| QS | Quorum Sensing |
| STING | Stimulator of Interferon Genes |
| TCP | Toxin Co-Regulated Pilus |
| TGF- β | Transforming Growth Factor Beta |
| Vh-CAI-1 | <i>Vibrio harveyi</i> Cholera Autoinducer-1 |
| WSCE | Water-Soluble Cranberry Extracts |
| WT | Wild-Type |

CHAPTER ONE

INTRODUCTION

I. COMMUNICATION BETWEEN BACTERIAL CELLS

Bacteria communicate with one another through hormone-like signals ¹⁻⁵. More specifically, bacterial cells produce chemicals called autoinducers for cell-cell communication, and this allows bacteria to behave in a specific manner depending on the signal ^{1, 3, 6, 7}. This cell-cell communication in bacteria is known as quorum sensing (QS). Quorum sensing was discovered in the 1990's, and relies on cell-density dependent signaling to aid in the survival and proliferation of bacteria ⁸. Autoinducers accompanied with acyl-homoserine lactones have been shown to regulate QS, and this was first observed in the mechanism of light production in *Vibrio fischeri* ⁹. QS has been studied extensively in Gram-negative bacteria, and many bacteria communicate with one another through the QS LuxR family of proteins ¹⁰. Some species have even been shown to elicit a response to these evolved QS molecules ¹⁰⁻¹².

II. INTERKINGDOM COMMUNICATION

Recently, there has been a lot interest in trying to understand the phenomenon that has been termed interkingdom communication. This is defined as the process in which bacteria and eukaryotes interact via small signaling molecules ^{2, 8}. Prokaryotes and eukaryotes have coexisted for millions of years, and numerous bacterial cells are present within humans, making up their endogenous bacterial flora. Humans have a symbiotic

relationship with their respective flora, but through signaling and other factors this relationship can either become detrimental (pathogens) or beneficial (probiotics). It has been shown that eukaryotes can detect these autoinducers, and this is vital for the success of the organism to locate food, avoid predators, etc.^{10, 13-22}. This process is known as chemotaxis, and it has been well studied in the nematode, *C. elegans*. Previous studies have looked at how *C. elegans* are able to sense and respond to specific autoinducers that are produced by different bacteria^{11, 23}. A study done by Zhang et al. (2012) showed that through the TGF- β pathways, DBL-1 is essential in *C. elegans* to actually learn and avoid the smell of certain pathogenic bacteria²⁴. Hasshoff et al. (2007) also showed that behavioral responses of *C. elegans* to avoid pathogenic strains of the Gram-positive *Bacillus thuringiensis* is dependent on the insulin-like signaling pathway, which is conserved in higher organisms including humans²⁵. Studying these interactions is thus important in understating how eukaryotes are able to detect and interpret environmental cues.

III. *VIBRIO* SPP. AUTOINDUCERS AND SECOND MESSENGERS

Vibrio spp. have been of particular interest to researchers, and they are widely viewed as model organisms for studying quorum sensing^{5, 11, 26}. *V. cholerae* is a Gram-negative rod-shaped bacterium that is mainly found in water, and is the causative agent of the disease cholera in humans¹¹. It is critical for this bacterium to communicate via quorum-sensing to persist and survive in the environment. Two autoinducers produced by *V. cholerae* have been characterized so far. The first is CAI-1, which is an intra-

species molecule, and the second is AI-2, which is inter-species⁸. CqsA and LuxS produce these autoinducers, respectively. This, along with other second messengers, allows *V. cholerae* to control biofilm formation, virulence, and other traits^{11, 18, 27, 28}. As well as causing disease in humans, this pathogen has also been shown to significantly decrease the lifespan of the model organism *C. elegans*, and kills them at a significant rate²⁹.

Numerous studies have been done studying the quorum-sensing mechanisms of *V. fischeri* and *V. harveyi*^{10, 17, 30}. These bacteria are known to have symbiotic relationships with various marine animals¹⁰. Unlike *V. cholerae*, they do not pose a threat to health for *C. elegans* and do not significantly decrease lifespan (unpublished data). *Vibrio* spp. have some conserved autoinducers and chemicals that they produce, but some species are unique. *V. harveyi* produces HAI-1, AI-2, and Vh-CAI-1, whereas *V. fischeri* produces AI-1, AI-2, and C8-HSL^{7, 11, 13, 28, 31}. As one can see, the autoinducers produced by *V. cholerae*, *V. fischeri*, and *V. harveyi* differ, and could be unique in communication. It has been shown that autoinducers produced by *V. cholerae* (in particular CAI-1) play a role in *C. elegans* chemoattraction¹¹. While this phenomenon is also observed in this experiment, it also appears that other signaling molecules could be having an effect. It is possible that other second messengers, such as cyclic di-nucleotides (CDNs), could be affecting the chemotaxis behavior of *C. elegans*.

Cyclic di-GMP (c-di-GMP) is one of the most prevalent intracellular signaling intermediates utilized by bacteria^{15, 16, 19}. It is known to play roles in biofilm formation and numerous receptors for c-di-GMP have been identified. A study recently completed

tried to elucidate the role of c-di-GMP in *V. cholerae* biofilm formation in the presence of water-soluble cranberry extract (WSCE). It was found that WSCE down-regulates genes involved in c-di-GMP synthesis and intracellular levels of c-di-GMP were thus significantly affected (unpublished data). Other CDNs have also been reported in other bacteria, specifically c-di-AMP. This molecule is known to function in Gram-positive bacteria by reporting on DNA integrity and cell membrane stress^{10, 15}. Remarkably, *V. cholerae* is able to produce a hybrid molecule of the two, termed c-GAMP²⁷. ToxT is a gene that activates the transcription of the virulence genes that are necessary for the pathogenesis of *V. cholerae*. In previous works, it has been shown that ToxT activity, acting through the TarB-VspR pathway, can cause derepression of DncV (VC0179), leading to increased levels of c-GAMP^{3, 19, 32}. This increase is linked to an inhibition of chemotaxis for *V. cholerae* and could aid in the stimulation of colonization and increase infectivity^{19, 32}. The proposed pathway can be seen in Figure 1.1.

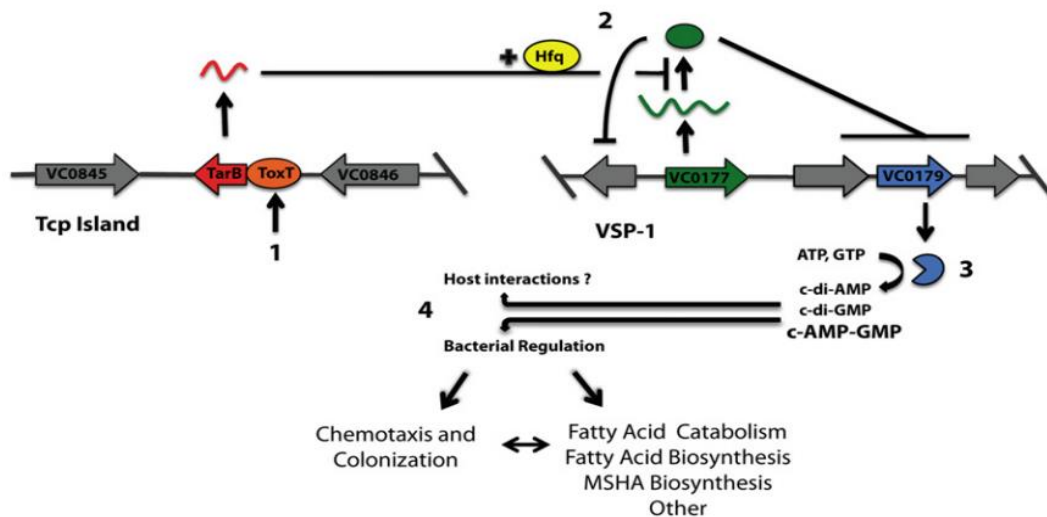


Figure 1.1. Proposed model for ToxT control of VSP-1 and DncV (VC0179)³².

IV. C. ELEGANS AS A MODEL SYSTEM

C. elegans are soil dwelling nematodes that grow to about 1 mm long, have a transparent body, and are capable of both sexual and asexual reproduction. Today, *C. elegans* is used in a variety of laboratory experiments to study cell signaling, gene regulation, ageing, etc.³³. This organism is of interest to researchers because they are easy to handle, have a low-cost compared to other model organisms, and have a relatively short lifespan (~14 days). They possess highly conserved molecular and cellular pathways, and its genome is surprisingly similar to that of humans (>30% homology)³³. *C. elegans* lacks an adaptive immune system, but many of the innate immune pathways, such as p38 MAPK, insulin-like signaling, TGF- β , etc., are also conserved in humans³.³³. Many *C. elegans* mutants are available for biological research, and this allows for extensive genetic studies to be conducted. Their genome is completely sequenced, and its neuronal network has been studied extensively. For these reasons, *C. elegans* is a great organism to use for my project.

V. SIGNIFICANCE

There are many bacteria that play an integral role in our lives, whether it is for better or worse. There are trillions of bacteria present within the gut of humans, and the commensal relationship we share with these bacteria is constantly fluctuating to find an appropriate balance. *V. cholerae* is readily able to form biofilms, establish in the gut, and cause infection in humans¹⁹. Autoinducers play a key role in the quorum sensing behavior of *V. cholerae*, and this signaling cascade controls virulence, biofilm formation,

and other traits ¹⁹. Second messengers like CDNs also serve as signaling molecules within prokaryotes, and it has recently been observed that humans and other animals can detect these molecules ³⁴. Studying the way in which these autoinducers and second messengers interact with a host could provide valuable information on how prokaryotes and eukaryotes are able to communicate as well as influence behavior. It is possible *V. cholerae* is using utilizing interkingdom communication to persist and spread throughout the environment. This study helps in better understanding the molecular processes involved in *V. cholerae* communication to survive, proliferate, and cause infection. We as humans rely on our senses, so this research could elucidate how humans and other animals perceive different cues within the environment and ultimately influence behavior within a host.

CHAPTER TWO

MATERIALS & METHODS

I. BACTERIAL STRAINS AND *C. ELEGANS* STRAINS

The bacterial strains used in this study are listed in Table 2.1. Dr. Jun Zhu graciously provided a number of the *V. cholerae* C6706 strains as well as the *V. harveyi* strain used in this study. *V. fischeri* strain was ordered from VWR (catalog# 470176-340), and *E. coli* OP50 came from the laboratory stock strain collection.

The *V. cholerae* and *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium. *V. harveyi* and *V. fischeri* were cultured in Marine Broth medium (Fischer). When performing inoculations for overnight cultures, streptomycin (100µg/ml) was used for antibiotic selection of all *V. cholerae* C6706 strains as well as *E. coli* OP50. No antibiotic selection was necessary for the growth of overnight cultures of *V. harveyi* and *V. fischeri*.

N2 worms were acquired from the Caenorhabditis Genetics Center (CGC). Worms were maintained on nematode growth medium (NGM) seeded with *E. coli* OP50 at 25°C, and transferred to fresh plates every two days.

Table 2.1. Strains used in this study

| Strain Name | Relevant Characteristics | Source |
|---|--|-----------------------------------|
| <i>V. cholerae</i> C6706 | | |
| (O1 El Tor) Strains | | |
| CO-15 | Wild Type | J. Zhu (10/26/2011) ³⁵ |
| CO-20 | PtcpA-lux reporter fusion (wild-type) | J. Zhu (7/3/2012) ³⁶ |
| CO-21 | toxT deletion with PtcpA-lux reporter fusion | J. Zhu (7/3/2012) ³⁶ |
| CO-23 | $\Delta cqsA$ | J. Zhu (7/1/2013) ³⁷ |
| CO-24 | $\Delta luxS$ | J. Zhu (7/1/2013) ³⁷ |
| CO-25 | $\Delta luxS/\Delta cqsA$ | J. Zhu (7/1/2013) ³⁷ |
| CO-36 | $\Delta dncV$ | B. Davies (2/2015) ³² |
| <i>V. harveyi</i> BB120 | | |
| CO-29 | None | Jun Zhu (3/14/2014) ³⁸ |
| <i>V. fischeri</i> (VWR# 470176-340) | | |
| CO-30 | None | Carolina Biological |
| Supply | | |
| <i>E. coli</i> (Laboratory Stock) | | |
| CE-71 | OP50 (Standard food for <i>C. elegans</i>) | Lab Stock |

II. CYCLIC DI-NUCLEOTIDES

Cyclic diadenosine monophosphate (c-di-AMP), cyclic diguanosine monophosphate (c-di-GMP), and cyclic adenosine monophosphate- guanine monophosphate (c-GAMP) were purchased from BioLog Life Science Institute. Their catalog numbers are C 088, C 057, and C 117, respectively.

Stock concentrations of these sodium salt compounds were made following the manufacturer's protocol. The stock samples were stored in -20°C freezer. Serial dilutions were performed from frozen stocks to obtain desired concentration before experiments were conducted.

III. PREPARATION OF MEDIA

NGM for maintenance was prepared via standard protocol in 60mm plates. *E. coli* OP50 was dropped on the center of the plates the night prior to transfer of worms. Bacterial strains were dropped onto the center of the plates 2 hours prior to worm transfer.

NGM-FUDR 35mm plates were used for lifespan assays. Fluorodeoxyuridine (FUDR) is an inhibitor of DNA synthesis, and at a concentration of 100µg/mL it prevents *C. elegans* from reproducing and doesn't interfere with development and aging post-maturation³⁹. Five times concentrated bacterial cultures were dropped in 100µL aliquots onto the center of plates 2 hours prior to worm transfer.

IV. CHEMOTAXIS ASSAY

C. elegans were grown at 25°C on *E. coli* OP50 under well-fed and un-crowded conditions. Chemotaxis assays were performed on standard LB agar plates. The plates were then divided in half to reveal the center point in each plate. Bacteria strains that were used for this experiment were grown in a shaking incubator overnight in the proper growth medium and temperature at approximately 100 rpm. Overnight cultures were then seeded onto each end of the plate 6cm apart, and a 2cm radius is drawn around each lawn. Refer to Figure 2.1 to see experimental setup of chemotaxis assay plate. The lawns are allowed to dry for 2 hours before experiments were conducted. After the lawns were able to dry, 1µL of 10mM sodium azide (NaN₃) was dropped on the center of each lawn to paralyze *C. elegans* to make sure they did not change bacterial lawns once one was initially chosen. Between 50 and 150 well-fed N2 worms were then placed in the center of the assay plate to begin the choice experiment. Every hour for 2 to 4 hours, worms present within the 2cm radius of each bacterial lawn were counted. Each assay was performed independently and in at least triplicate. Choice index was calculated as follows:

$$\textbf{*Choice Index} = (\textbf{\# of worms on Test} - \textbf{\# of worms on Control}) / \textbf{Total \# of worms}^{24}.$$

***Positive values indicate preference towards Test strain, while negative values indicate preference towards Control**

Standard deviation is calculated for the experiments so variance can be observed. Graphs and calculations were performed in Microsoft Excel. Given the choice index calculation, if no preference is observed in the experimental procedure then the equation would yield a value of 0. To determine if preference is significant towards the Test or

Control lawn, choice index calculated from experimental procedure was compared to 0 (no preference) by conducting a t-test. If the choice index was to be analyzed further between bacterial strains or time points, then t-test were calculated further between the trials. After statistical analysis, a P-value <0.05 was accepted as statistically significant between variables.

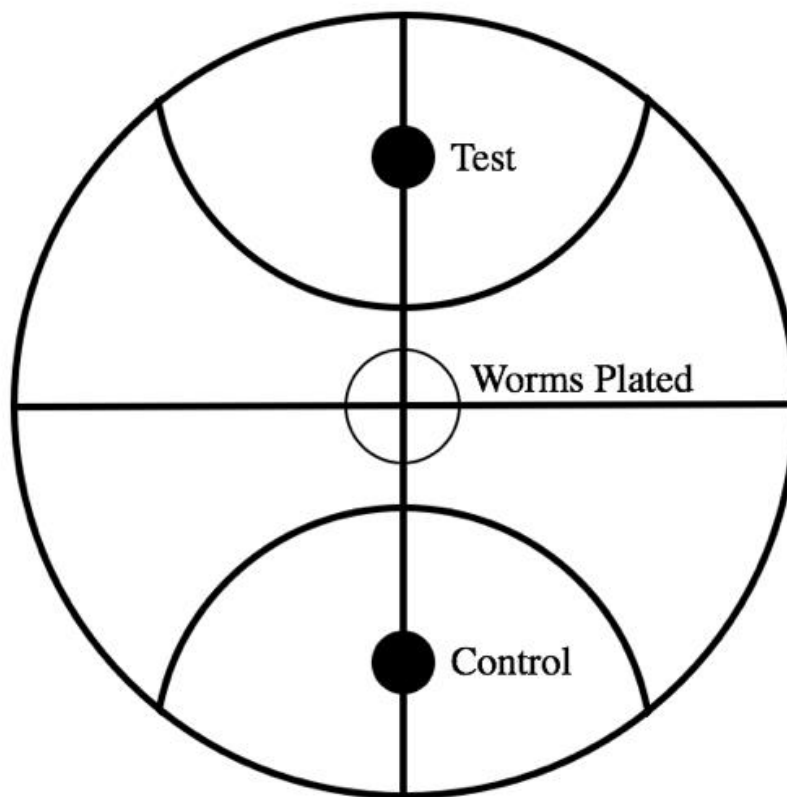


Figure 2.1. Diagram of experimental setup for chemotaxis assay plates.

V. SUPPLEMENTATION WITH CYCLIC DI-NUCLEOTIDES

For some of the chemotaxis assays conducted, proper bacterial strains were inoculated with a certain concentration of the CDNs mentioned in Part II. This was done to test whether supplementation with these compounds changed the chemotactic response of *C. elegans* in their presence. Since no previous tests have been done regarding this experiment, a range of concentrations was used when testing these molecules (0.1nM, 1nM, 5nM, and 10nM). These concentrations were prepared from the stock by serial dilutions before each experiment was conducted.

Bacterial colonies that were tested were grown overnight as previously described in a shaking incubator. The cultures were then directly supplemented with the appropriate CDN at the appropriate final concentration in the bacterial solution. This solution was then dropped onto chemotaxis assay plates and allowed to dry in the previously described manner. Assays were then completed as previously described and choice index was determined after successful completion.

VI. LIFESPAN ASSAYS

Lifespan assays were carried out at 25°C. Worms were synchronized by transferring 20 gravid worms to 60mm NGM plates seeded with *E. coli* OP50 2 days prior to the start of assays. Worms were allowed to lay eggs for 5 hours, and then parent worms were removed from plates, leaving only synchronized eggs. Worms were then incubated until L4 stage. Overnight bacterial cultures were concentrated by centrifugation and removal of 50% of supernatant. Cultures were then resuspended in remaining

medium. Aliquots of 50 μ L of *V. cholerae* strains and 100 μ L of *E. coli* OP50 were dropped on the center of NGM-FUDR 35mm plates and allowed to dry for 2 hours. Worms in L4 stage were transferred to assay plates; 20 worms per plate. Plates were subsequently checked daily and dead worms were counted and recorded. Day of transfer was defined as day zero. Statistical analysis was carried out through SPSS software under the Kaplan-Maier lifespan analysis. P-values were determined via log rank test, and $P < 0.05$ was accepted as statistically significant.

CHAPTER THREE

RESULTS

I. *C. ELEGANS* N2 PREFER *V. CHOLERA* WILD-TYPE OVER *E. COLI* OP50

Through initial experiments conducted in the laboratory, it was noticed that *C. elegans* were strongly attracted to *V. cholerae* C6706. This was particularly interesting because *V. cholerae* has been shown to kill *C. elegans* at a high rate, and significantly decreases lifespan. It is hypothesized that the decrease in lifespan of *C. elegans* could be due to the production of different autoinducers by *V. cholerae*. This could influence behavior in the *C. elegans* model. For this reason, studies were completed to observe the chemotaxis behavior in *C. elegans* when exposed to *V. cholerae* wild-type (WT) strain. Figure 3.1 shows that N2 worms readily attracted towards *V. cholerae* WT compared to its normal laboratory food, *E. coli* OP50. Chemotaxis assays were conducted in which the behavior and preference of *C. elegans* was observed over the course of 4 hours. This is a standard laboratory experiment to test preference in the nematode model³². These experiments were conducted in triplicates with standard deviation calculated to obtain error bars. There was no significance difference in Choice Index between the hours studied.

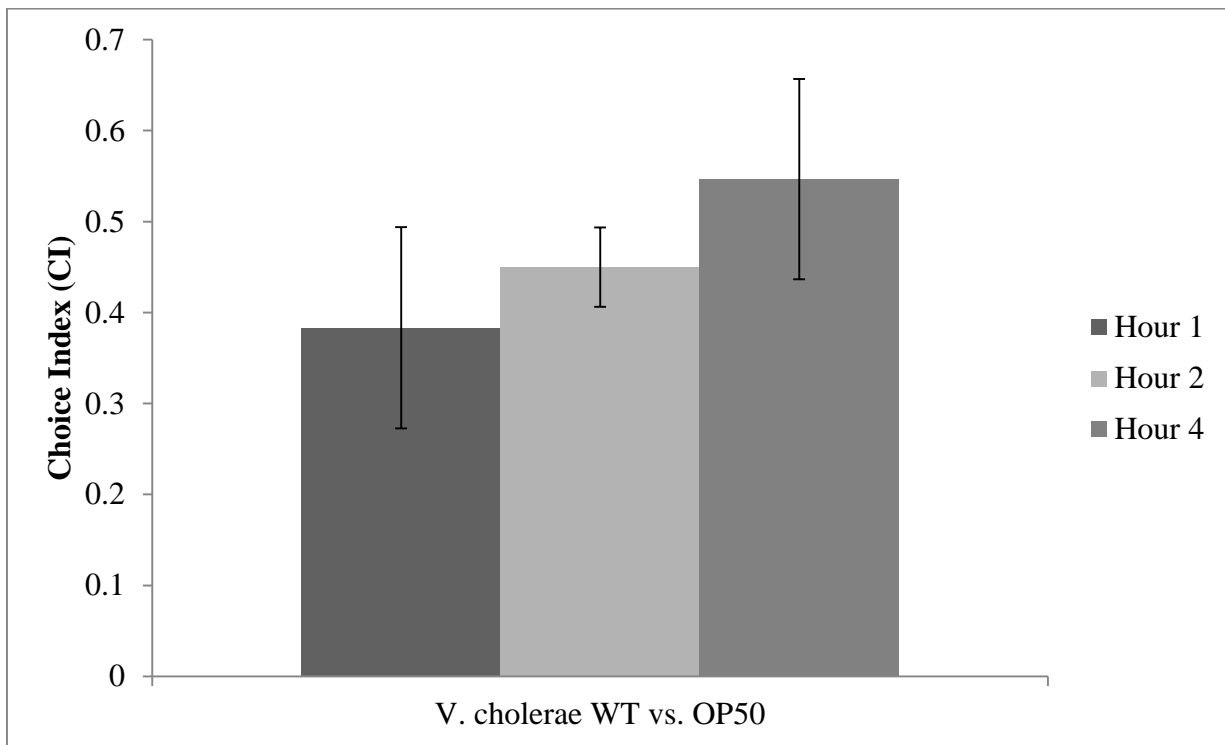


Figure 3.1. Choice Index calculations showing preference of *C. elegans* N2 towards *V. cholerae* C6706 WT over *E. coli* OP50 over the course of 4h. All choice indexes calculated had $p < 0.001$ when compared to choice index = 0.

II. TWO AIs ARE INVOLVED IN *C. ELEGANS* CHEMOTACTIC RESPONSE TOWARDS *V. CHOLERAE*

Based on the results from Figure 3.1, it was of interest to test *V. cholerae* WT as a control against three mutant strains ($\Delta cqsA$, $\Delta luxS$, $\Delta luxS/\Delta cqsA$) unable to produce one or both autoinducers, and observe the chemotaxis behavior of *C. elegans*. Based on the choice index calculated, it was found that *C. elegans* showed strong preference towards *V. cholerae* WT when compared to the other three mutant strains (Figure 3.2). Preference towards the WT over the double mutant strain, $\Delta luxS/\Delta cqsA$, which is unable to produce any autoinducers, was significantly higher than preference observed towards the other two mutant strains.

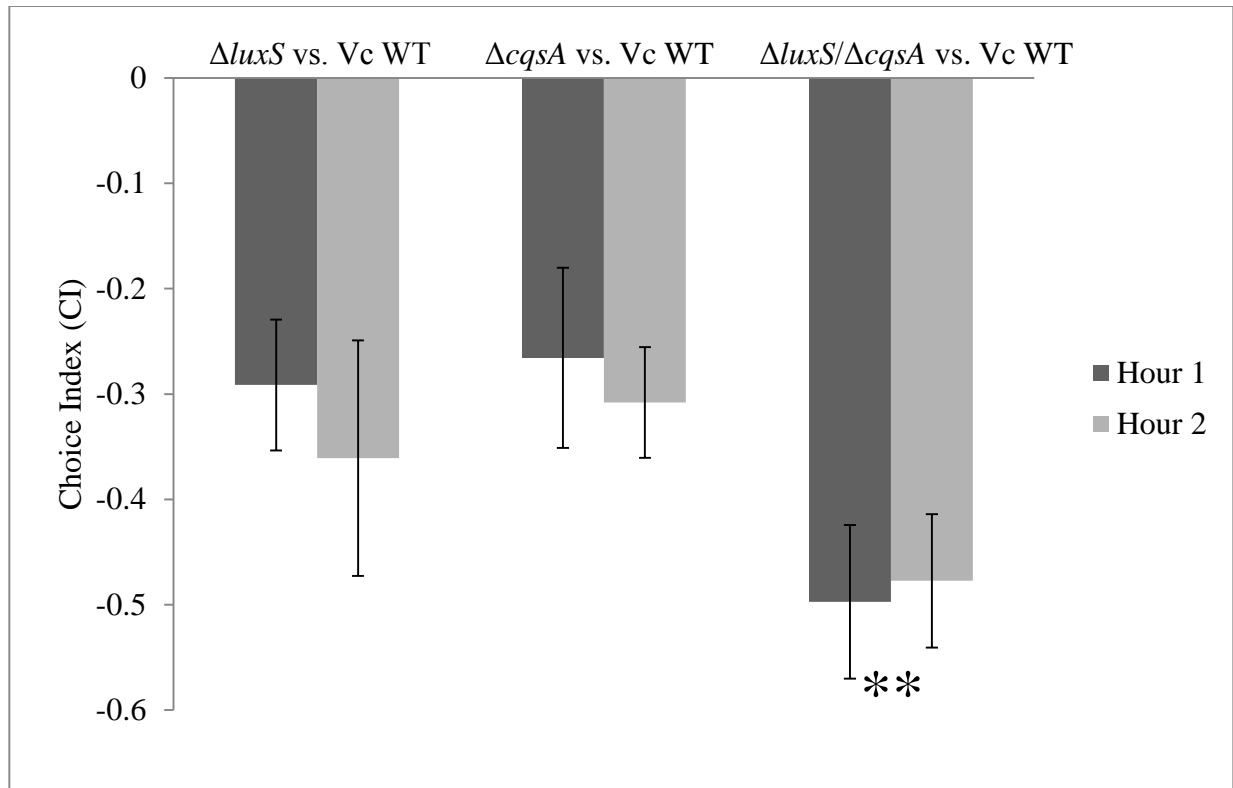


Figure 3.2. Choice Index calculations showing preference of *C. elegans* N2 towards *V. cholerae* C6706 WT (control) over deletion mutant strains of *V. cholerae* C6706, $\Delta cqsA$, $\Delta luxS$, $\Delta luxS/\Delta cqsA$ (test). All choice indexes calculated had $p < 0.001$ when compared to choice index = 0. ** indicates $p < 0.001$ calculated between choice indexes for each trial.

III. *C. ELEGANS* CAN SENSE VARIOUS AI MOLECULES, WITH PREFERENCES

Knowing that AIs are playing a role in chemotaxis, it prompted investigation of the chemotaxis behavior of *C. elegans* when allowed to choose between two other non-pathogenic *Vibrio* strains when compared to the pathogenic *V. cholerae*. The autoinducers produced by *V. fischeri* and *V. harveyi* are different from *V. cholerae*, as well as each other. Through preliminary experiments, it was found that these *V. fischeri* and *V. harveyi* strains do not significantly decrease the lifespan of *C. elegans*, which is noted in the presence of *V. cholerae* (data not shown). Chemotaxis assays were carried out, and preference was observed towards both *V. fischeri* and *V. harveyi* (Figure 3.3). The production of autoinducers in *V. fischeri* and *V. harveyi* is most likely playing a role in the health and behavior of *C. elegans*. Preference towards *V. fischeri* was evident, but the standard deviation was large, whereas preference of *C. elegans* towards *V. harveyi* was strong and conclusive.

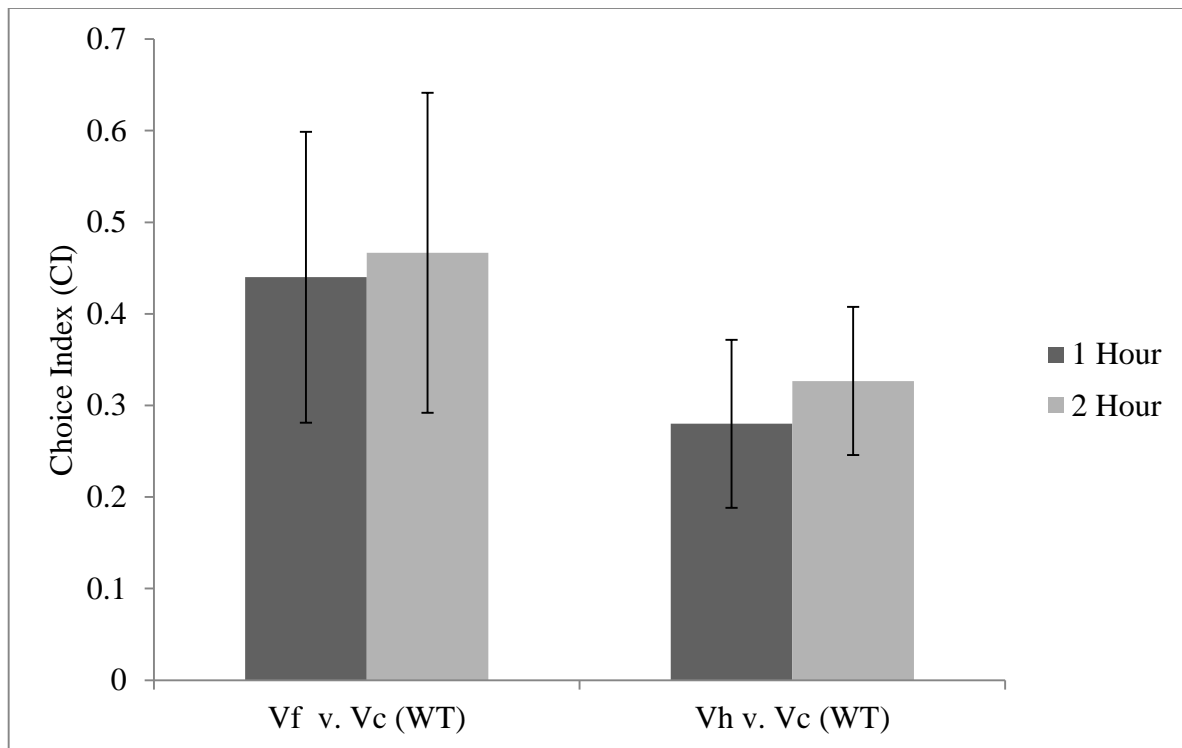


Figure 3.3. Choice Index calculations showing preference of *C. elegans* N2 towards *V. fischeri* and *V. harveyi* when tested against *V. cholerae* C6706 WT as a control. All choice indexes calculated had $p < 0.001$ when compared to choice index = 0.

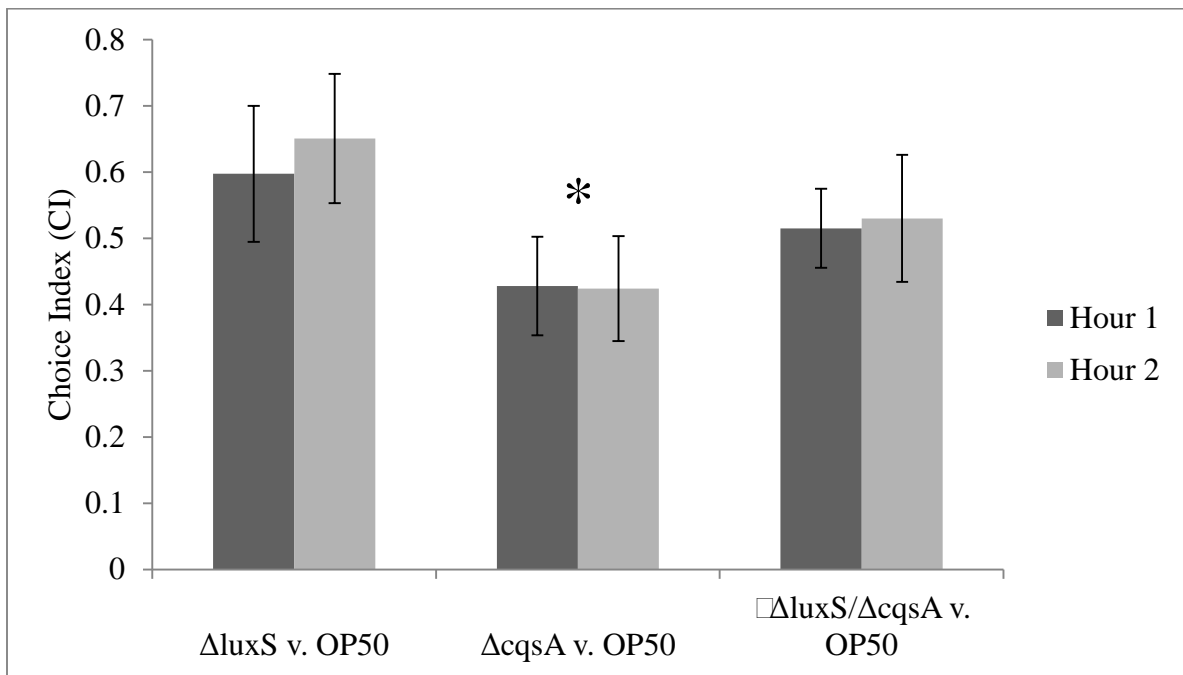
IV. OTHER SIGNALING MOLECULES APPEAR TO BE INVOLVED IN CHEMOTAXIS.

From the results obtained in Figure 3.2, it was seen that *C. elegans* was able to prefer *V. cholerae* WT over mutant strains that were unable to produce autoinducers. It was then of interest to test these mutant strains ($\Delta cqsA$, $\Delta luxS$, $\Delta luxS / \Delta cqsA$) against *E. coli* OP50 to observe if preference was dependent on the production of autoinducers.

In Figure 3.4-A, the three mutant strains were tested against *E. coli* OP50 as a control. *C. elegans* N2 preference was observed towards all three mutant strains. Preference towards $\Delta luxS$ was significantly higher when compared to the other two strains tested, while preference towards $\Delta cqsA$ was the lowest. On the other hand, preference is still observed towards the other two mutants conveying the notion that other molecules are also playing a role.

Of note in Figure 3.4-A, there is preference towards C6706 $\Delta luxS / \Delta cqsA$ strain (DM) over *E. coli* OP50 even though there is no autoinducers being produced. To determine if this preference was caused by something produced extracellularly by the pathogen, overnight cultures of the *V. cholerae* C6706 strains were centrifuged and the supernatant was then added to overnight inoculum of *E. coli* OP50. This bacterial solution was then seeded onto chemotaxis assays plates, and tested against a normal overnight culture of *E. coli* OP50 as a control. The results can be found in Figure 3.4-B, and preference can still be seen towards *V. cholerae* strains over *E. coli* OP50. Preference towards $\Delta luxS / \Delta cqsA$ leads to the possibility that other signaling molecules produced by *V. cholerae* can be sensed by *C. elegans*.

A



B

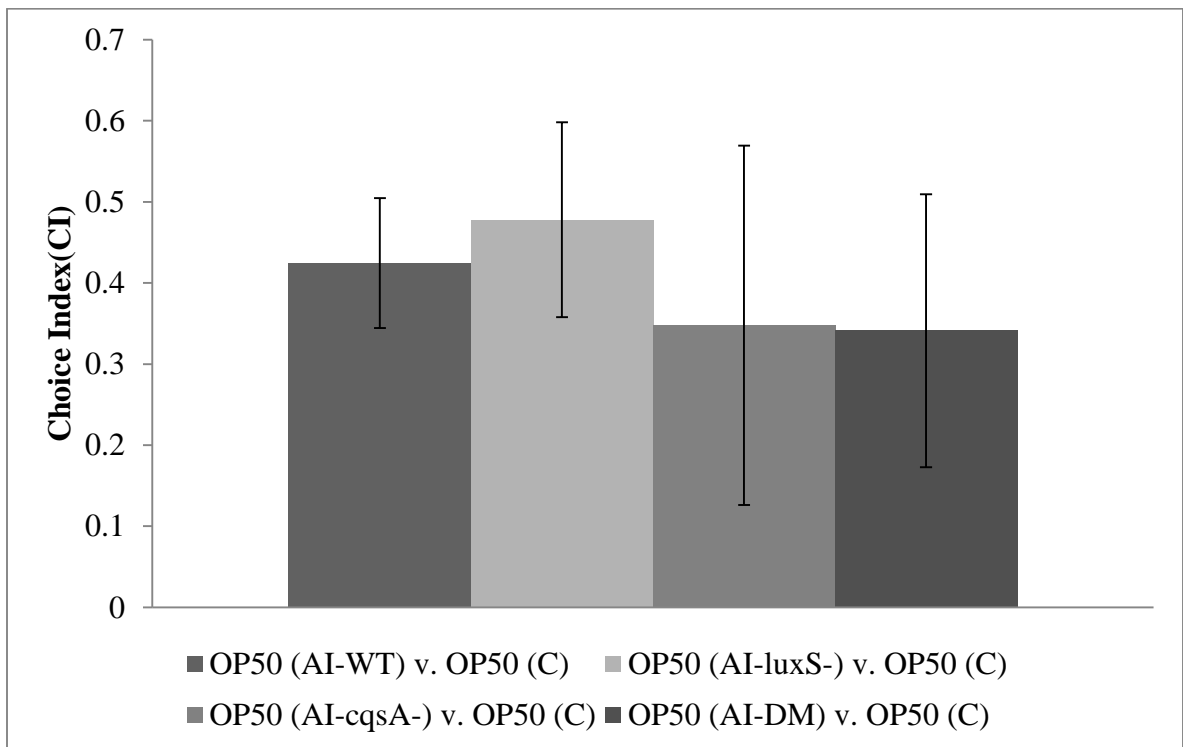


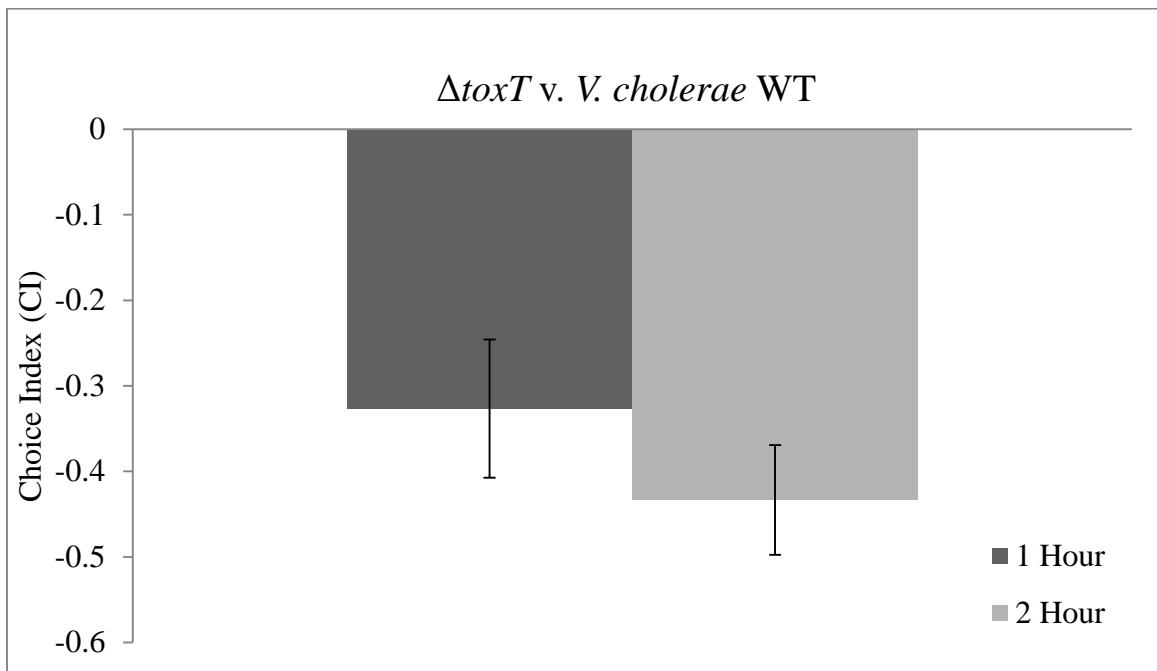
Figure 3.4. Choice Index calculations showing preference of *C. elegans* N2. **A)** Preference towards *V. cholerae* C6706 mutant strains ($\Delta cqsA$, $\Delta luxS$, $\Delta luxS/\Delta cqsA$) over *E. coli* OP50 (control). **B)** Preference towards *E. coli* OP50 supplemented with supernatant of *V. cholerae* C6706 overnight cultures (test) over standard *E. coli* OP50 (control). All choice indexes calculated had $p < 0.001$ when compared to choice index = 0. * indicates $p < 0.05$ calculated between choice indexes for each trial.

V. POSSIBLE ROLE OF CDNs IN CHEMOTAXIS. *C. ELEGANS* N2 PREFERENCE
TOWARDS *V. CHOLERA* SEEMS TO BE DEPENDENT ON ToxT, AND MORE
SPECIFICALLY, DncV

As stated earlier, ToxT activates the transcription of the virulence genes that are necessary for the pathogenesis of *V. cholerae*. Its activation also leads to increased levels of the hybrid molecule c-GAMP, as well as smaller amounts of c-di-GMP and c-di-AMP in vitro. Thus studying the chemotactic response of *C. elegans* by testing *V. cholerae* $\Delta toxT$ in comparison to the WT would be beneficial to observe if attraction towards *V. cholerae* is influenced by the production of these second messengers. The results in Figure 3.5-A show a strong preference of *C. elegans* N2 towards *V. cholerae* WT over *V. cholerae* $\Delta toxT$ (control). Preference appears to be dependent on the proper functioning of ToxT and production of c-GAMP, and possibly c-di-GMP and c-di-AMP.

Since *C. elegans* preference toward *V. cholerae* seems to be dependent on ToxT, further tests were conducted to see if the di-nucleotide cyclase that is unique to *V. cholerae*, DncV, is also playing a role. This cyclase lies downstream of the ToxT cascade and is directly responsible for the production of c-di-AMP, c-di-GMP, and the dominant molecule c-GAMP. Chemotaxis assays were conducted testing preference of *C. elegans* against *V. cholerae* $\Delta dncV$ and *V. cholerae* WT as a control. The results can be found in Figure 3.5-B, and a strong preference towards *V. cholerae* WT was noted. Based on these results, the hypothesis is further reaffirmed that *C. elegans* preference towards *V. cholerae* is dependent on these genes, as well as the production of CDNs.

A



B

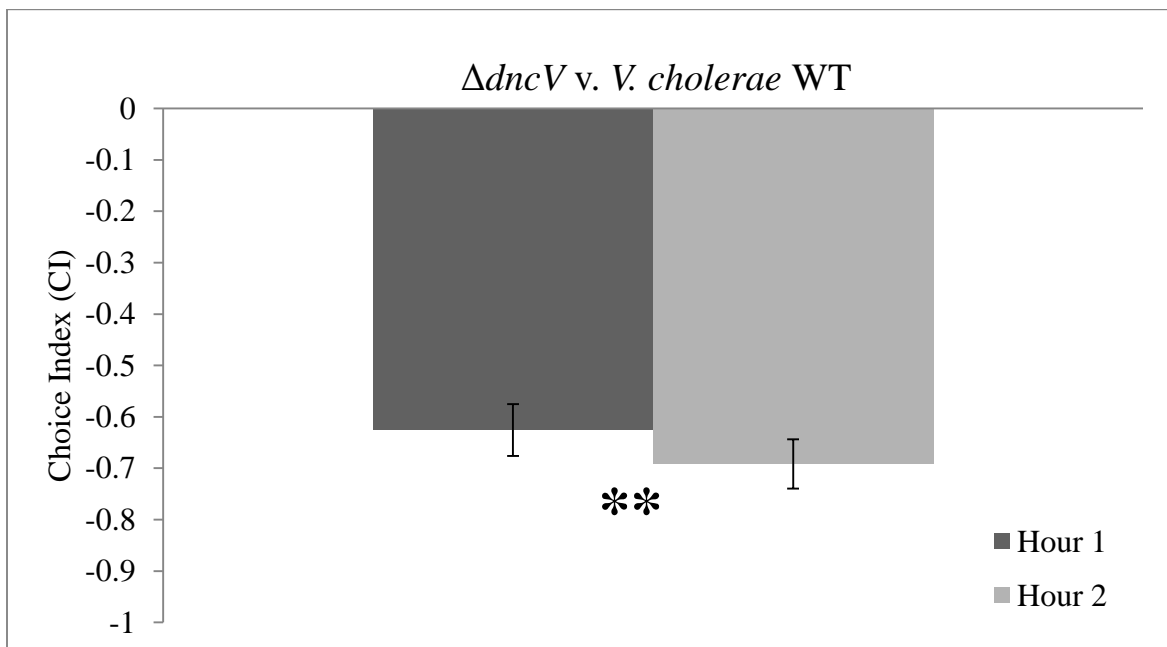


Figure 3.5. Choice Index calculations showing preference of *C. elegans* N2. **A)**

Preference towards *V. cholerae* C6706 WT when tested against *V. cholerae* $\Delta toxT$. **B)**

Preference towards *V. cholerae* C6706 WT when tested against *V. cholerae* $\Delta dncV$. All

choice indexes calculated had $p < 0.001$ when compared to choice index = 0. ** indicates

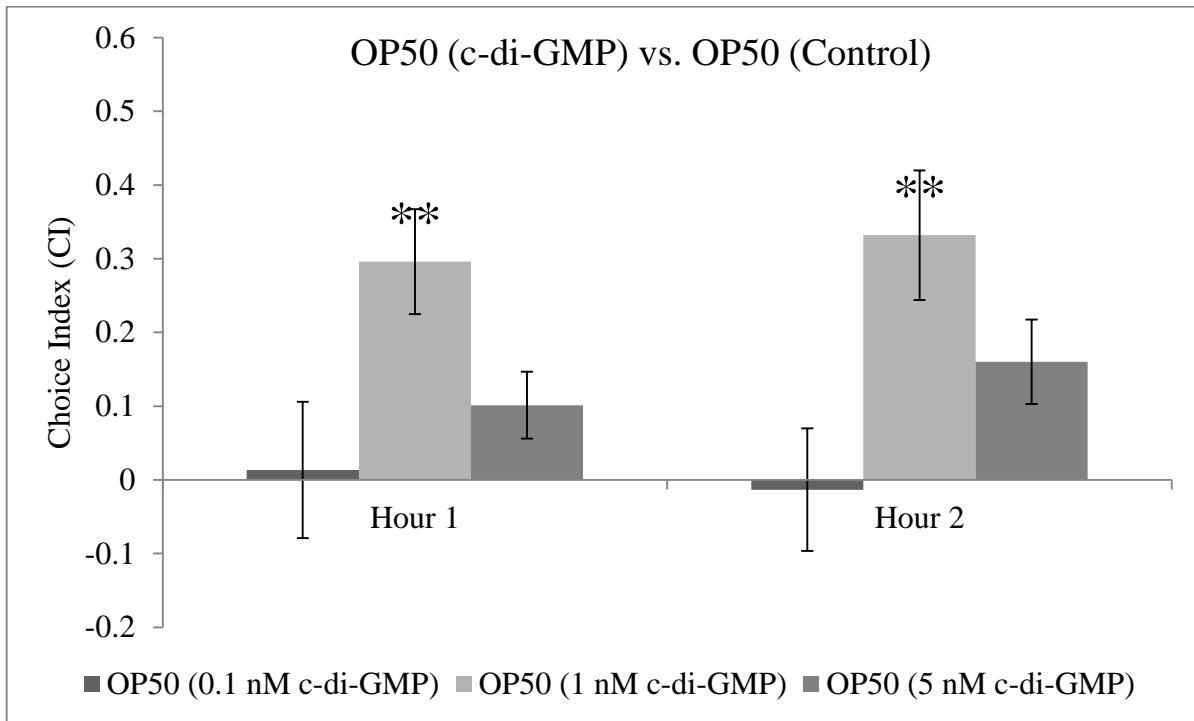
$p < 0.001$ calculated between choice indexes for each trial ($\Delta toxT$ v. *V. cholerae* WT

compared to $\Delta dncV$ v. *V. cholerae* WT)

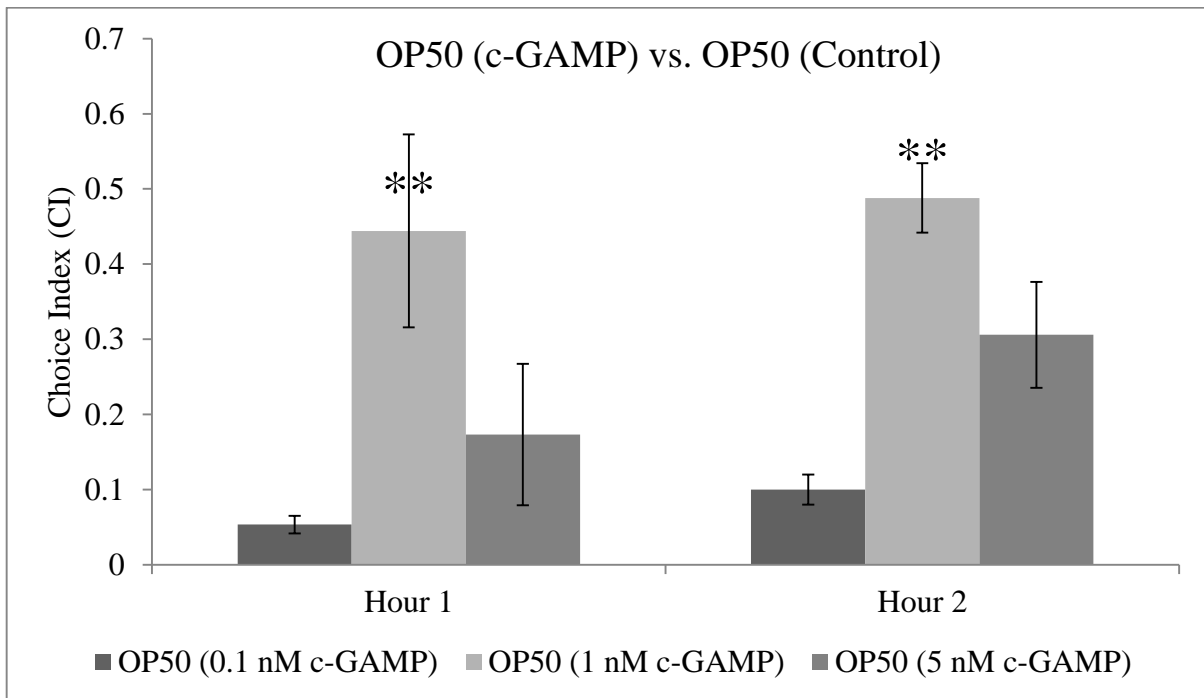
VI. *C. ELEGANS* N2 CAN SENSE C-GAMP AND C-DI-GMP AT AN OPTIMAL CONCENTRATION OF 1nM

It seems that if second messengers like the CDNs seem to be driving chemoattraction (Figure 3.5), but at what concentrations can *C. elegans* sense these molecules if at all? To test this, pure solutions of c-di-GMP, c-GAMP, and c-di-AMP were purchased. Chemotaxis assays were conducted as normal, except *E. coli* OP50 was supplemented with different concentrations of the CDNs to observe preference. Figure 3.6-A and B shows that 1nM of both c-di-GMP and c-GAMP are able to cause a strong attractive behavior in *C. elegans*. There was a stronger chemotactic response seen toward the *E. coli* OP50 supplemented with c-GAMP. However, as shown in Figure 3.6-C, this same preference isn't noticed when tested with c-di-AMP, and the worms seem to be repulsed by the presence of this molecule. This is interesting in that preference seems to be influenced by both c-di-GMP and c-GAMP, and not c-di-AMP ruling out the possibility that maybe preference by *C. elegans* could just be influenced by the presence of DNA. 0.1nM concentration of c-di-AMP was not tested because this concentration did not produce much of a response based on the results from Figure 3.6-A and B.

A



B



C

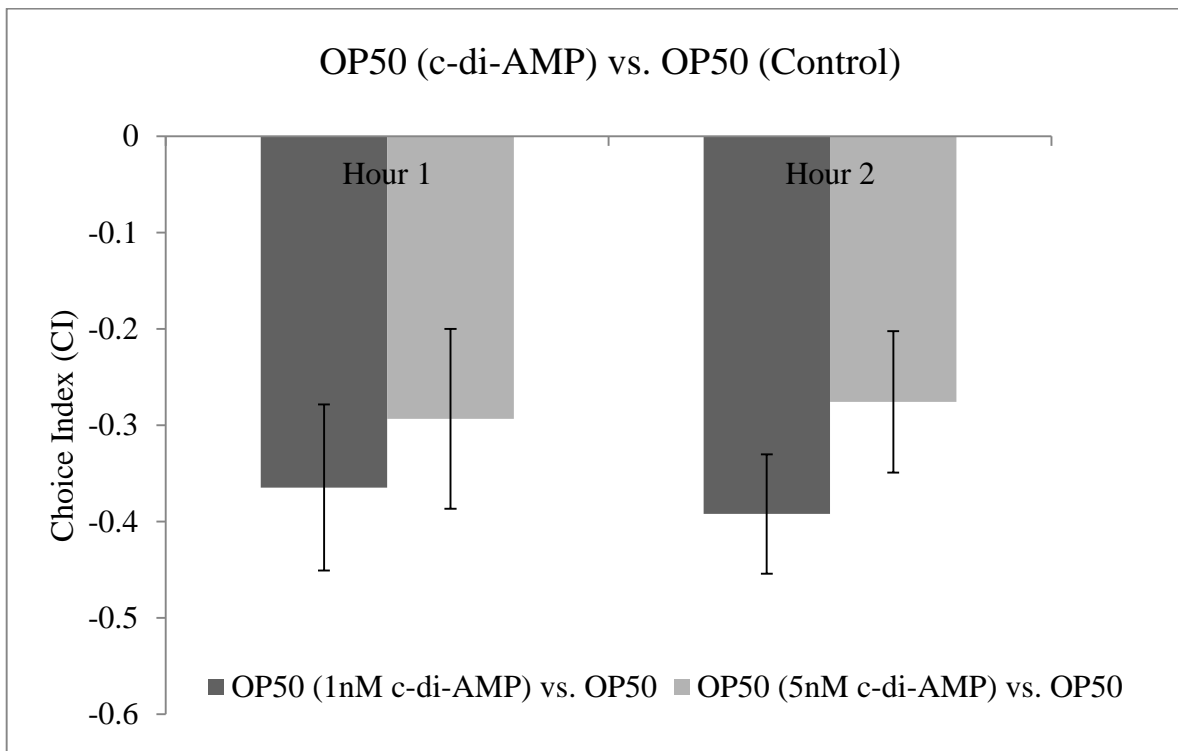


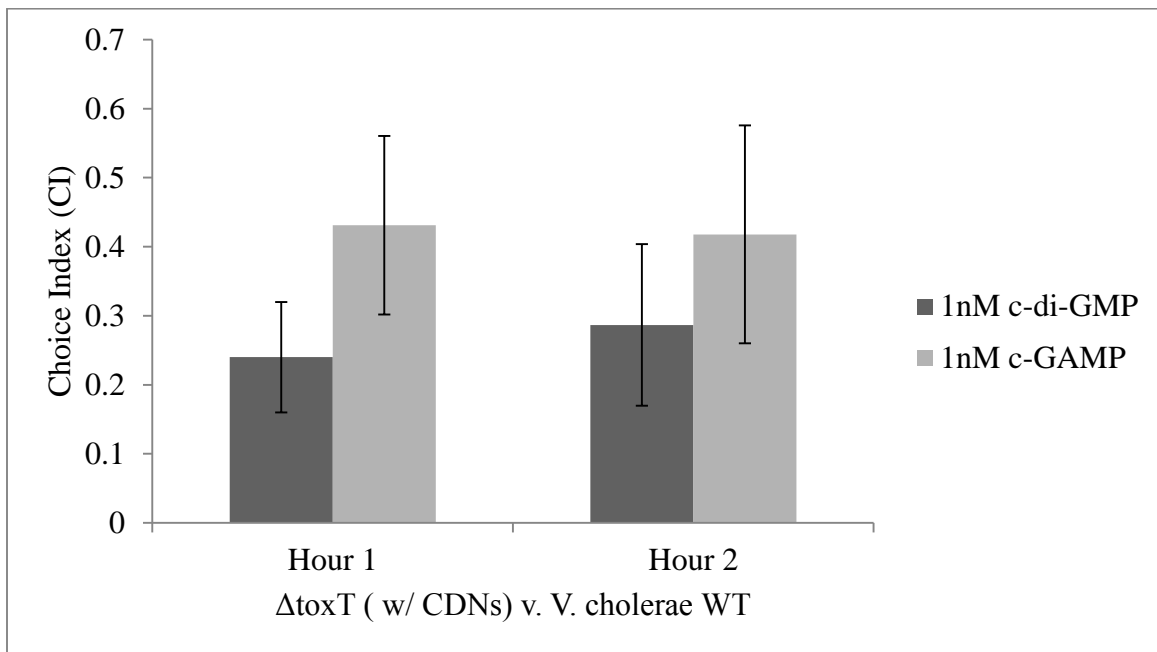
Figure 3.6. Chemotaxis index showing preference of *C. elegans* N2. **A)** Preference towards *E. coli* OP50 supplemented with c-di-GMP at an optimal concentration of 1nM. **B)** Preference towards *E. coli* OP50 supplemented with c-GAMP at an optimal concentration of 1nM. **C)** Preference towards *E. coli* OP50 (control) over *E. coli* OP50 supplemented with c-di-AMP. All choice indexes calculated had $p < 0.001$ when compared to choice index = 0. ** indicates $p < 0.001$ calculated between choice indexes for each trial.

VII. SUPPLEMENTATION OF C-DI-GMP AND C-GAMP AT 1nM RESTORES

ATTRACTIVE CHEMOTAXIS OF *C. ELEGANS* N2

Referring back to Figure 3.5, it is noted that when ToxT and DncV are not functioning in *V. cholerae*, *C. elegans* is not able to sense these bacterial lawns and readily prefer the normal *V. cholerae* WT strain. To test if this response could be reversed, supplementation of c-di-GMP and c-GAMP at a concentration of 1nM were added to $\Delta toxT$ and $\Delta dncV$ overnight cultures and chemotaxis assays were conducted. What was found is very interesting in that when these mutants were supplemented with 1nM concentrations of both c-di-GMP and c-GAMP, chemoattraction of *C. elegans* was seen towards the lawns supplemented with the CDNs (Figure 3.7-A,B). *C. elegans* N2 also had a significant preference towards 1nM of c-GAMP compared to 1nM of c-di-GMP when supplemented in both *V. cholerae* $\Delta toxT$ and *V. cholerae* $\Delta dncV$.

A



B

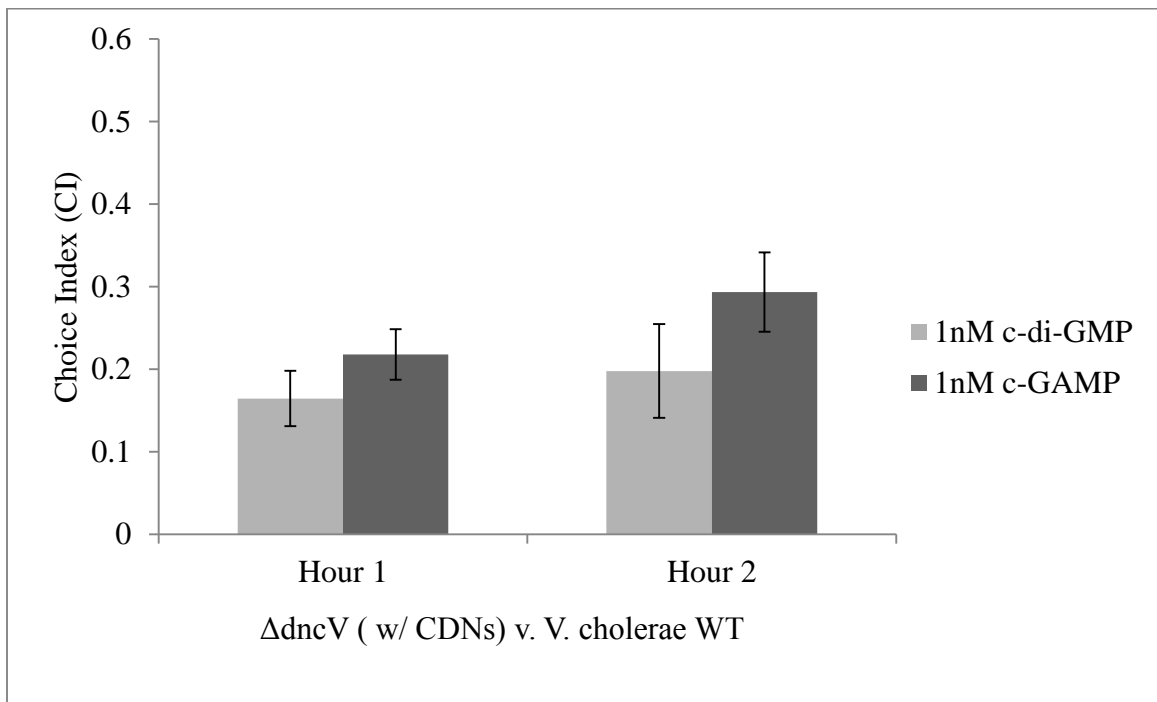


Figure 3.7. Chemotaxis index showing preference of *C. elegans* N2. **A)** Preference towards *V. cholerae* $\Delta toxT$ supplemented with 1nM c-di-GMP and 1nM c-GAMP over *V. cholerae* WT. **B)** Preference towards *V. cholerae* $\Delta dncV$ supplemented with 1nM c-di-GMP and 1nM c-GAMP over *V. cholerae* WT. All choice indexes calculated had $p < 0.001$ when compared to choice index = 0.

VIII. *V. CHOLERAE* KILLING OF *C. ELEGANS* N2 IS DEPENDENT ON ToxT AND PARTIALLY DEPENDENT ON DncV

ToxT lies upstream of the cyclase DncV, and controls many genes that are involved in the virulence of *V. cholerae*. To try and better understand if these genes play a role in the pathogenicity of *V. cholerae* within *C. elegans* N2, lifespan assays were conducted. *E. coli* OP50, the normal laboratory food of *C. elegans*, was used as a control and *V. cholerae* WT, *V. cholerae* $\Delta dncV$, and *V. cholerae* $\Delta toxT$ were tested. Figure 3.8 and Table 3.1 show the results and statistical analysis of the completed lifespan assays. The lifespan of *C. elegans* N2 was significantly decreased in the presence of *V. cholerae* WT and *V. cholerae* $\Delta dncV$ when compared to *E. coli* OP50 and *V. cholerae* $\Delta toxT$. Interestingly, the lifespan of *C. elegans* N2 was not significantly changed when grown in the presence of *V. cholerae* $\Delta toxT$ compared to *E. coli* OP50. This suggests that ToxT is required for the pathogenesis of *V. cholerae* in *C. elegans*. Also of note, when compared to *V. cholerae* WT, *C. elegans* N2 grown in the presence of *V. cholerae* $\Delta dncV$ lived significantly longer although not nearly as long as those grown in the presence of *E. coli* OP50 and *V. cholerae* $\Delta toxT$. All lifespan assays were conducted independently and in triplicate.

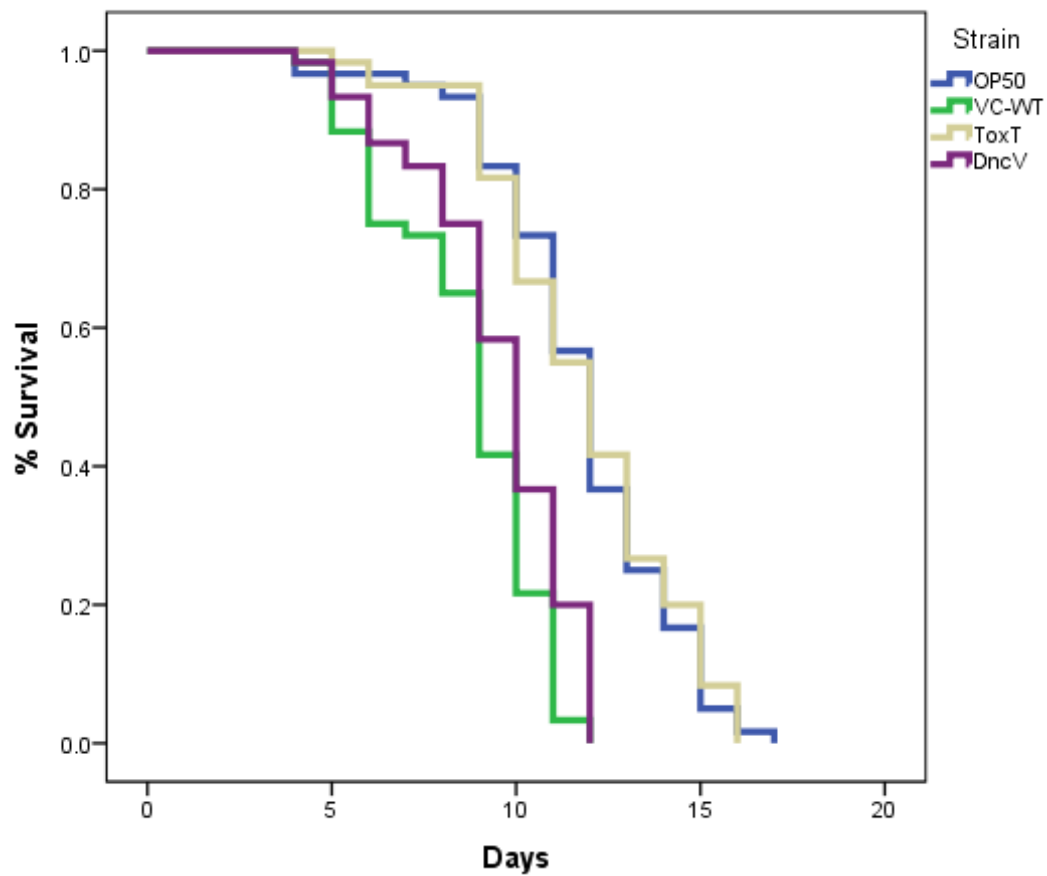


Figure 3.8. *C. elegans* N2 lifespans are decreased significantly in the presence of *V. cholerae* WT and *V. cholerae* $\Delta dncV$ at 25°C. *V. cholerae* $\Delta toxT$ has no significant impact on lifespan when compared to *E. coli* OP50.

Table 3.1. Effect of *V. cholerae* C6706 WT and mutant strains on lifespan on *C. elegans* N2 at 25°C.

| Strain | Mean±SE (Days) | N | P-value |
|---|----------------|----|---|
| <i>E. coli</i> OP50 | 11.767±0.336 | 60 | -- |
| <i>V. cholerae</i> WT | 8.667±0.276 | 60 | <0.05 ^a |
| <i>V. cholerae</i> Δ <i>toxT</i> | 11.833±0.337 | 60 | 0.808 ^a , <0.05 ^b |
| <i>V. cholerae</i> Δ <i>dncV</i> | 9.517±0.275 | 60 | <0.05 ^a , 0.008 ^b |

^a compared to *E. coli* OP50, ^b compared to *V. cholerae* WT

CHAPTER FOUR

DISCUSSION

I. *C. ELEGANS* CAN SENSE AUTOINDUCERS PRODUCED BY *V. CHOLERAE*

Vibrio cholerae is able to communicate and survive through quorum sensing, in which the bacterium produces autoinducers to relay information. *V. cholerae* is able to produce two autoinducers, CAI-1 which is an intra-species molecule, and AI-2 which is inter-species. CqsA and LuxS are responsible for producing these molecules, respectively. The production of autoinducers is believed to control many important traits in *V. cholerae* such as biofilm formation and virulence^{40, 41}. Recently, it has been shown that *C. elegans* can detect the autoinducer CAI-1 produced by *V. cholerae* through the AWC^{ON} sensory neuron¹¹. However, this phenomenon is not only confined to the production of this single molecule.

Through the experimental procedure, it was well noted that *C. elegans* was able to sense and prefer *V. cholerae* WT over its normal laboratory food (Figure 3.1). This sparked interest to determine which molecules were driving chemoattraction, thus the production of autoinducers was initially studied. Figure 3.2 shows that when *luxS*, *cqsA*, or both genes are knocked out there is a significant preference towards the WT strain over these mutants. There is no significant difference between the choice indexes calculated for $\Delta luxS$ and $\Delta cqsA$, meaning that the production of each autoinducer (AI-2 and CAI-1) can be sensed and responded to by *C. elegans*. When both autoinducers are not being produced ($\Delta luxS / \Delta cqsA$), *C. elegans* chooses the WT significantly greater than that observed when tested against the single mutants. So both of these autoinducers are

playing a role, but can *C. elegans* detect other autoinducers produced by different *Vibrio* species?

V. harveyi is able to produce three autoinducers, HAI-1, AI-2, and Vh-CAI-1, whereas *V. fischeri* produces AI-1, AI-2, and C8-HSL^{7, 11, 13, 28, 31}. When *C. elegans* was able to choose between these two *Vibrio* species compared to *V. cholerae*, they significantly preferred *V. fischeri* and *V. harveyi* (Figure 3.3). From these results, it appears that *C. elegans* can sense and respond to the autoinducers produced by *V. fischeri* and *V. harveyi*. These two *Vibrio* species have been known to have symbiotic relationships with marine organisms, and they also do not affect the health or lifespan of *C. elegans* N2 (data not shown). It is possible that *C. elegans* can detect these autoinducers to overcome the pathogenicity associated with *V. cholerae*. Although there is preference seen towards *V. cholerae* over its normal laboratory food, this data suggests that there is an order of preference associated with the detection of *Vibrio* autoinducers. To understand the order of preference in *V. cholerae* more chemotaxis assays were conducted, and the results were very interesting.

Figure 3.4-A shows that *C. elegans* significantly prefers *V. cholerae* mutant strains ($\Delta cqsA$, $\Delta luxS$, $\Delta luxS/\Delta cqsA$) over *E. coli* OP50. The greatest preference was observed towards *V. cholerae* $\Delta luxS$, and preference was significantly lower towards $\Delta cqsA$ and $\Delta luxS/\Delta cqsA$. These findings support the work done by Werner et al., which explains that CAI-1 (produced by CqsA) is the main autoinducer in *V. cholerae* sensed by *C. elegans*¹¹. On the contrary, there is still significant preference towards *V. cholerae* $\Delta luxS/\Delta cqsA$, in which no autoinducers are being produced. Even when only the

supernatant of these *V. cholerae* mutants were tested against *E. coli* OP50, preference was still significantly seen towards the mutant strains (Figure 3.4-B). There must be other molecules produced by *V. cholerae* other than autoinducers that are influencing the chemoattractive behavior of *C. elegans*.

II. C-DI-GMP AND C-GAMP ARE INFLUENCING BEHAVIOR OF *C. ELEGANS* TOWARDS *V. CHOLERA*E

To be successful, *V. cholerae* must be able to establish within the gut of a host and correctly utilize many transcription factors to influence virulence^{42, 43}. CDNs are important second messengers, and as research advances new discoveries are being made about how they affect and influence many cellular pathways^{16, 32, 44-49}. CDNs have been studied extensively in bacteria, but new roles are being observed and studied in eukaryotes^{15, 44}. C-di-GMP and c-di-AMP have been an area of interest for a number of years, and have different roles in Gram-negative and Gram-positive bacteria respectively⁵⁰. In *V. cholerae*, c-di-GMP has been shown to regulate things such as virulence factors, flagellum biosynthesis, and biofilm formation⁵¹⁻⁵³. C-di-AMP is more prevalent in Gram-positive bacteria and can function to report on DNA integrity, cell membrane stress, and can play a role in establishing bacterial infection within a host^{10, 15, 54, 55}. Recently, a novel cyclase has been identified in *V. cholerae*, DncV, that is able to synthesize the hybrid molecule c-GAMP³². In this study, it was hypothesized that CDNs produced by *V. cholerae* are the underlying molecules that are aiding in the communication between this bacterium and a eukaryotic host.

ToxT has been referred to as the master regulator of virulence in *V. cholerae*, and this is achieved through the activation of genes that encode the cholera toxin (CT) and toxin co-regulated pilus (TCP), which aids in surface attachment^{32, 56, 57}. ToxT acts through the TarB-VspR pathway, and causes derepression of DncV leading to increased levels of c-GAMP^{3, 19, 32}. Chemotaxis of *V. cholerae* is then inhibited and hyperinfectivity ensues as a result of increased colonization within the gut.

To test the role of ToxT and DncV in *C. elegans* attraction towards *V. cholerae*, chemotaxis assays were completed testing deletion mutants ($\Delta toxT$ and $\Delta dncV$) against the WT. Figure 3.5 shows that when these genes are knocked out, *C. elegans* significantly prefers and chooses the WT strain when given the choice. This leads to the conclusion that these two genes are required by *V. cholerae* to obtain a chemoattractive response from *C. elegans*. Preference of *C. elegans* towards *V. cholerae* WT over $\Delta dncV$ is also significantly greater than preference towards *V. cholerae* WT over $\Delta toxT$. Through the proposed model³², DncV should not be functioning if ToxT is deleted from the strain, so theoretically the choice indexes should not be significantly different. Thus it appears that other factors or pathways are able to regulate DncV and ultimately the production of c-GAMP. This evidence is supporting of the hypothesis that CDNs are playing a role in communication with a host.

In a study done by Davies et al., in vitro studies revealed that DncV is able to produce c-di-GMP and c-di-AMP, but preferentially produces substantially more of the hybrid molecule, c-GAMP³². Solutions of these three compounds were purchased and used to test the behavioral response of *C. elegans* in the presence of these molecules at

varying concentrations. Based on previous studies testing the role of c-di-GMP in *V. cholerae* biofilm formation, lower concentrations ranging from 0.1nM-5nM were tested for these molecules (unpublished data). The results in Figure 3.6-A and B show that *C. elegans* N2 is able to sense and respond to c-di-GMP and c-GAMP at different concentrations when added to a culture of *E. coli* OP50. Based on calculations, the optimal concentration for detection of these two molecules seems to be around 1nM. A concentration of 0.1nM did not result in a significant behavioral response from the test worms. Because of this, c-di-AMP concentrations were only tested at 1nM and 5nM. Interestingly, the results from Figure 3.6-C show that *C. elegans* significantly chooses *E. coli* OP50 that has not been supplemented with any pure concentrations of c-di-AMP. This indicates that *C. elegans* may not be able to sense this molecule, or the more plausible theory that they can sense it and avoid the bacterial lawn purposefully.

Since *C. elegans* is able to sense c-di-GMP and c-GAMP, tests were then conducted by supplementation of these molecules in to *V. cholerae* $\Delta toxT$ and $\Delta dncV$ mutants at 1nM concentrations. These treated strains were then used in chemotaxis assays to determine if *C. elegans* is able to choose these treated mutant strains over *V. cholerae* WT. Previously it was shown that *C. elegans* significantly chooses the WT over $\Delta toxT$ and $\Delta dncV$ mutants (Figure 3.5), but when these mutant strains are treated with 1nM of c-di-GMP and 1nM of c-GAMP preference switches significantly towards the deletion mutants (Figure 3.7). Of note, preference of *C. elegans* towards $\Delta toxT$ and $\Delta dncV$ that were supplemented with 1nM of c-GAMP elicited a significantly greater choice index when compared to $\Delta toxT$ and $\Delta dncV$ supplemented with 1nM c-di-GMP.

From these results, it suggests that c-GAMP is more easily sensed by *C. elegans* and produces a stronger behavioral response.

III. ToxT AND DncV INFLUENCE *V. CHOLERAE* PATHOGENESIS

The initial choice and attraction of *C. elegans* to preferentially feed on *V. cholerae* has a significant adverse effect on the health of the organism (Figure 3.8) ²⁹. It has been seen that the production of autoinducers as well as second messengers by *V. cholerae* drives *C. elegans* chemoattraction towards the pathogen. But do these molecules and genes that produce them have an effect on the health of a host? ToxT controls the transcription of many virulence factors in *V. cholerae*, and the function of DncV is only known to produce c-GAMP *in vivo* and possibly c-di-GMP and c-di-AMP at low concentrations, but this has not been proven. To test the role of these genes on the health of *C. elegans*, lifespan assays were conducted using *E. coli* OP50 (normal laboratory food strain), *V. cholerae* WT, *V. cholerae* Δ toxT, and *V. cholerae* Δ dncV.

Referring to Figure 3.8 and Table 3.1, *V. cholerae* WT decreases the lifespan on *C. elegans* N2 significantly when compared to *E. coli* OP50. The mean days of survival decreases from 11.767 to 8.667. Compared to the WT, *C. elegans* N2 grown in the presence of *V. cholerae* Δ toxT and *V. cholerae* Δ dncV lived significantly longer. The mean days of survival were 11.833 and 9.517, respectively. However, worms grown on the *V. cholerae* Δ dncV strain experienced a significant decrease in lifespan compared to OP50. Interestingly, *V. cholerae* Δ toxT strain did not have a significant effect on the lifespan of *C. elegans* N2 compared to OP50 (p-value 0.808). These findings are

important because they reveal that *V. cholerae* killing of *C. elegans* is dependent on ToxT, and partially dependent on DncV. To our knowledge, this is the first time that this dependency has been reported.

Given the function of ToxT and the virulence factors it regulates, it is not a surprise that the killing of *C. elegans* relies on the proper functioning of this regulon. Through ToxT, genes encoding the cholera toxin (*ctxAB*) and TCP proteins are transcribed, including a number of different colonizing factors ⁵⁸. However, CT and TCP have been shown to not be necessary for the pathogenesis of *V. cholerae* in *C. elegans* and humans. In a recent study, CT and TCP negative *V. cholerae* strains are still able to cause disease and it is shown that a hemolysin, *hlyA*, is required for lethal infection of *V. cholerae* in *C. elegans* ⁵⁹. Also, through the HapR regulon, which is important in *V. cholerae* quorum-sensing cascade, a protease (PrtV) is needed for the successful killing of *C. elegans* ⁶⁰. In the proposed model, DncV is regulated by the ToxT regulon, and derepression of the cyclase causes a decrease in *V. cholerae* chemotaxis allowing for greater colonization and infectivity ³². When *toxT* is removed, killing of *C. elegans* is not observed, and in theory *dncV* is still under repression. In contrast, when *dncV* is deleted killing is still observed, but to a lesser extent than the WT. This supports the hypothesis that second messengers, such as c-GAMP and c-di-GMP, are playing a role in the pathogenesis of *V. cholerae* and possibly the immune response of a host. It also seems that DncV is under regulation by other factors that are still unknown.

IV. FUTURE DIRECTIONS

From the results obtained, it is obvious that autoinducers produced by *V. cholerae* are playing a role in the chemoattraction of *C. elegans*, but it seems that other second messengers are influencing behavior as well. The cyclic di-nucleotides produced by *V. cholerae*, specifically c-GAMP and c-di-GMP are very interesting molecules, and their roles in different processes and communication pathways are not well defined. They could be some of the key molecules that allow for communication between prokaryotes and eukaryotes.

To further define the role of DncV in communication with a host, testing a triple mutant in which no autoinducers or c-GAMP is being produced ($\Delta luxS / \Delta cqsA / \Delta dncV$) would be useful. Utilizing plasmid construction, overexpression of DncV can also be established in *E. coli* OP50 or *V. cholerae* $\Delta toxT$ to further elucidate the behavioral response of *C. elegans* to CDNs. Knowing that CAI-1, AI-2, c-di-GMP, and c-GAMP can be sensed in some way by *C. elegans*, establishing an order of preference through further tests would be very beneficial in determining which molecules have a more significant role in communication. Of course, if CDNs are shown to be influencing behavior significantly within a host, detection of these molecules will have to be confirmed in the supernatant of cultures to prove they are being produced extracellularly. Quantification of these molecules in the supernatant could thus reveal precise concentrations in which these molecules are being detected.

Since the signaling molecules detected by *C. elegans* have been identified, it is vital to understand precisely how these molecules are being detected. *C. elegans* has been

shown to detect CAI-1 through the AWC^{ON} neuron, but the detection of the other signaling molecules have not been defined¹¹. Through a bioinformatics study and literature review, potential genes in *C. elegans* have been chosen to test the binding of *V. cholerae* signaling molecules.

After these steps have been completed, research will shift to focus on how the host is able to respond to these bacterial signals. Although preliminary results have already been obtained through lifespan assays (Figure 3.8 and Table 3.1), more experiments can be conducted to obtain a better understanding. *V. cholerae* is not the only pathogen that can be sensed by *C. elegans*. The behavioral avoidance of pathogens by an organism is an effective technique, but through preliminary studies *C. elegans* does not show the ability to learn and avoid *V. cholerae* (data not shown). *V. cholerae* O1 El Tor may have evolved over time to further refine communication to persist and spread easily throughout the environment.

The detection of bacterial nucleic acids is critical for the activation of the innate immune system in humans and other animals. There is a transmembrane protein known as STING (stimulator of interferon genes) in humans that is important in viral or bacterial nucleic acid detection, and is found to bind to c-di-GMP produced from bacterial infections^{15, 19}. The hybrid c-GAMP is also able to bind to STING and stimulate interferon gene expression¹⁹. To the best of our knowledge, there is no information available on STING in *C. elegans*, but many of the pathways involving innate immunity are conserved between *C. elegans* and humans (p38 MAPK, ILS, TGF- β , etc.). Therefore

through utilizing qRT-PCR, it would be of interest to test genes expression levels involved in *C. elegans* innate immunity.

This research will hopefully shed some light on the growing area of research of interkingdom communication. Knowing the specific way in which a host is able to sense and respond to certain signals has a broad impact on the behavioral aspects of different organisms. Studying this could also reveal novel mechanisms or pathways that pathogenic bacteria use to cause disease within a host, which in turn allows for development of therapeutic agents or new intervention strategies.

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